Effects of Electric Stimulation on Bovine Oocyte Activation and Embryo Development in Intracytoplasmic Sperm Injection Procedure

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Purpose: *This study was carried out to investigate the efficacy of electric stimulation before and/or after intracytoplasmic sperm injection (ICSI) on bovine oocyte activation and embryo development.*

Methods: *The oocytes were treated with electric shock before (B), before and after (B&A), and after (A) sperm injection. In each group, sham ICSI (ICSI-s) was performed to exclude the effect of parthenogenesis (B ICSI-s, B&A ICSI-s, and A ICSI-s). An electric pulse was applied with a single direct current (DC) pulse (0.8 kV/cm, 70 μsec).*

Results: *One pronucleus (PN) formation in the B&A ICSI-s group was slightly higher than that found in B and B&A ICSI group; however, the difference was not significant. Two PN formation in B&A ICSI group was higher than that found in sham ICSI groups ($P < 0.05$). There were no differences among treatment groups in the cleavage rate; however, morulae and blastocyst formation in the B&A embryos was significantly higher than that of other groups ($P < 0.05$) and got pregnant.*

Conclusions: *Electric stimulation before and after injection was an effective method in inducing bovine oocyte activation and in sustaining embryo development to the morulae and blastocyst stage.*

KEY WORDS: Bovine oocyte; electric stimulation; embryo development; intracytoplasmic sperm injection; pregnant.

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INTRODUCTION

Oocyte activation is a series of events triggered by the fertilizing spermatozoon and is essential for the beginning of embryonic development. However, the mechanism of oocyte activation is still poorly understood. Moreover, there is some concern on how oocyte activation proceeds after intracytoplasmic sperm injection (ICSI), which bypasses the normal sequence of fertilization events, such as penetration of zona pellucida (ZP) and fusion between the plasma membrane of both gametes (1).

Calcium has an important role in the events of egg activation and early development. Human oocytes fertilized after ICSI can develop a calcium response similar to normal fertilization, but the beginning of the sperm-induced calcium response is always considerably delayed after ICSI (2). Edwards and Van Steirteghem (3) hypothesized that oocytes are activated by the injection of high calcium medium during ICSI procedure. The activation of an oocyte is then initiated not by the ICSI procedure but by a sperm-associated oocyte-activating factor (SAOAF) released from the injected spermatozoon (1, 4). However, bovine ICSI is very different from that of other species. In the bovine ICSI procedure, low fertilization rates have been reported. Fertilization rates were improved significantly by artificial stimulation using calcium ionophore A23187 (Ca ionophore) or ethanol (5–8). The most effective method, however, for the activation of bovine oocyte has not yet been established. Therefore, it is important to develop a more efficient activating method of bovine oocyte ICSI in order to obtain higher fertilization and development results. The objective of the present study was to evaluate the effect of electric stimulation before and/or after sperm injection on bovine oocyte activation and embryo development.

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MATERIALS AND METHODS

Collection and Maturation of Oocytes

The ovaries were obtained from Korean native cows (Hanwoo) at a local slaughterhouse and were brought to the laboratory in physiological saline (0.9% NaCl) at 37°C within 3 hr. Cumulus–oocyte complexes were collected by aspirating follicles 5–8 mm in diameter. These were cocultured on granulosa cell monolayer with culture medium (TCM199 supplemented with 10% fetal calf serum) at 39°C, 5% CO_2 incubator for 22–24 hr.

Granulosa cells (GCs) were obtained from large follicles (≥20 mm) that were not contaminated with red blood cells and were centrifuged at 700 × *g* for 5 min. The supernatant was decanted. The GC pellet was resuspended with culture medium (3 ml) and centrifuged two times at 300 × *g* for 5 min. Then the pellet was diluted to 1×10^6 cells/ml concentrations. Several GC microdrops (50 μl) were placed on the culture dish (3001, Falcon) and covered with mineral oil (M-8410, Sigma, USA). The dishes were left at 39°C, in a 5% CO_2 incubator for 1–2 hr until oocytes transferred.

Preparation of Cryopreserved Semen

Cryopreserved Hanwoo bull semen was thawed by placement of the straws at room temperature for 10 sec and then plunged into 37°C water bath for 1 min. Sperm washing was performed by diluting semen with 5 ml (Brackett and Oliphant) medium (9) containing 10 mM caffeine and centrifuged at 700 × *g* for 5 min. After two centrifugations, the supernatant was discarded and the final pellet was gently resuspended with 1 ml BO medium supplemented with 10 μg ml heparin, 5 mM caffeine, and 3 mg/ml bovine serum album (BSA). The sperm were allowed to swim up for 1–2 hr at 39°C, 5% CO_2 incubator.

Intracytoplasmic Sperm Injection

Matured bovine oocytes were treated with hyaluronidase (1 mg/ml, type IV-S, Sigma, USA) for 3 min, and the cumulus cells surrounding oocyte were gently removed by pipetting. Injection of the spermatozoa was carried out as described by Goto *et al.* (10). Briefly, the oocyte was held by holding pipette [inner diameter (ID) 10–20 μm, outer diameter (OD) 140–150 μm] and the injection pipette (ID 7–8 μm, OD 8–9 μm) containing a single spermatozoa was introduced deeply into the oocyte cytoplasm, its membrane having first been broken by cytoplasm aspiration. The spermatozoon was injected into the ooplasm with a small amount of medium. Prior to injection, each individual spermatozoon was immobilized by damaging the plasma membrane of the midpiece by exerting pressure with the tip of the injection pipette.

Treatment for Oocyte Activation

The denuded oocytes were activated using three methods of electrical stimulation. The first method delivered an electric stimulation at 30–90 min before (B) injection. The second method delivered an electric pulse 30–90 min before and after (B&A) microinjection. The last method delivered the electric pulse at 30–90 min after (A) injection. Each sham ICSI group was treated with the same electric stimulation without sperm injection. The electric stimulation procedure was as follows. The oocytes were placed in a chamber filled with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.05% BSA for 5 min equilibration. After the equilibration time, the five to six oocytes were moved into an electrode wire chamber (0.5 mm apart) and aligned with the electrode by 6 V of alternating current (AC). Electric stimulation of oocytes was applied using a single direct current (DC) pulse (0.8 kV/cm, 70 μsec; Electric Cell Manipulation, BTX2001, USA) in the same solution. All the activated oocytes were washed three times and transferred into the culture medium drop.

Assessment of Pronucleus Formation

To assess pronucleus (PN) formation, about one third of the treated embryos were mounted on glass slides at 17–18 hr after stimulation, fixed with fixative solution (acetic acid: methanol = 1:3) for less than 24 hr, and then stained with 0.3% acetic lacmoid.

In Vitro Development

The last treated zygotes were cocultured with bovine oviductal epithelial cells in 20 μl culture media microdrop. Half of culture medium was replaced with new medium every 48 hr. Assessment of embryonic development was recorded at medium exchange.

Embryo Transfer

Two embryos that developed to blastocyst stage obtained 7 days after sperm injection were nonsurgi-

cally transferred to the uteri of synchronized recipient cattle. Pregnancy diagnosis was by ultrasound and rectal palpation after 48 days.

Statistical Analysis

The PN formation and the proportions of embryos developed to ≤ 4-cell and blastocyst stages were analyzed using Fisher's exact test. Differences below the 0.05 probability level ($P < 0.05$) were considered statistically significant.

RESULTS

The PN formation rate of ICSI and sham ICSI group embryos are shown in Table I. Although 1PN formation rates in the sham ICSI groups were slightly higher than ICSI groups; however, the difference was not statistically significant. Particularly significant differences were noted in the B&A ICSI group embryos compared to sham ICSI groups in 2PN formation ($P < 0.05$). Three PN formation SD was noted in B&A ICSI-s and A ICSI groups, but the difference was not significant. There were no differences in the cleavage rates between each group; however, the developmental rate to morulae and blastocyst stages of B&A ICSI group was significantly higher than that of other groups ($P < 0.05$), except A ICSI group (Table II). One of the two transferred blastocysts obtained from B&A ICSI group became pregnant and has been ongoing until now (6 months).

DISCUSSION

In all animal species, the fertilization process initiates a cascade of events in the oocyte, which is termed "oocyte activation." During normal fertilization, fusion of the membranes is followed by the unification of oocyte and sperm cytoplasm. It is reasonable to assume that during or following this fusion process, a SAOAF is released into the ooplasm (11). During ICSI, however, membrane fusion is bypassed, and how oocyte activation proceeds is unclear. Swann *et al.* (12) reported that damage to the sperm plasma membrane prior to injection would be necessary to allow a SAOAF to leave a spermatozoon.

Unlike other species, the activation and cleavage rate of sperm-injected bovine oocytes are low (6, 10, 13, 14). A major cause of this low development rate is that bovine oocytes cannot be successfully activated by the simple injection of spermatozoon including cytoplasm aspiration into injection pipette (5). Previous experiments in our laboratory had reconfirmed that ICSI-only bovine oocytes failed to attain cleavage (unpublished data).

Recently, artificial stimulation to accelerate an increase of calcium in the ooplasm has demonstrated an improvement in the rate of oocyte activation (15). The mechanism, however, by which artificial stimuli generate oocyte activation is unclear. It had been suggested that increases in cytoplasmic calcium at fertilization are important for the resumption of the cell cycle in all species examined (16, 17). Oocytes are normally arrested at metaphase of meiosis II at the time of ovulation, due to cytostatic factor, which counteracts the activity of stabilized meiosis promoting factor. Calcium elevation by artificial stimulation induces an inactivation or destruction of cytostatic factor, resulting in a resumption of meiosis event (18).

The Ca ionophore or ethanol has been used to stimulate oocyte activation and embryo development. When injected oocytes are activated by 10-min incubation in 50 μM of Ca ionophore before and after injection, cleavage rates are 21–38% and 2.5–6.7% of the

Table I. Pronucleus Formation of Injected Oocytes by Electric Stimulation

Treatment		No. of oocytes[a]	Pronucleus formation (Mean ± SD)		
			1 PN	2 PN	3 PN
Before	ICSI	25	4 (16 ± 7.7)	9 (36 ± 9.8)	
	ICSI-s	24	7 (29.2 ± 7.2)	4 (16.7 ± 7.2)	
Before and after	ICSI	28	4 (14.3 ± 6.7)	16 (57.1 ± 11.4)[b]	
	ICSI-s	28	12 (42.9 ± 8.5)	2 (7.1 ± 6.4)	2 (7.1 ± 6.4)
After	ICSI	26	6 (23.1 ± 10.4)	10 (38.5 ± 11.2)	1 (3.8 ± 3.6)
	ICSI-s	28	7 (25 ± 7.1)	4 (14.3 ± 5.1)	2 (7.1 ± 5.7)

[a] Three sets of replications.
[b] B and A ICSI was significantly higher on 2 PN formation than other groups.

Table II. Development of Treated Embryos Treated with Electric Stimulation

Treatment		No. of oocytes[a]	No. of cleavage (Mean ± SD)	No. of embryos developed to (%)		
				≤4-cell	5- to 16-cell	M + B
Before	ICSI	48	16 (35.4 ± 3.6)	12 (25)	3 (6.3)	1 (2.1)
	ICSI-s	44	13 (29.5 ± 5.8)	8 (18.2)	5 (11.4)	0 (0.0)
Before and after	ICSI	46	20 (47.8 ± 7.3)	7 (15.2)	7 (15.2)	6 (13.0)[b]
	ICSI-s	45	18 (40 ± 7.9)	6 (13.3)	12 (26.7)	0 (0.0)
After	ICSI	52	18 (34.6 ± 5.3)	9 (17.3)	7 (13.5)	2 (3.8)
	ICSI-s	45	11 (26.7 ± 4.7)	3 (6.7)	7 (15.6)	1 (2.2)

[a] Three sets of replications.
[b] Was higher than B on M + B.

embryos developed to the blastocyst stage (5–7, 19). Others have shown that 2.2% of bovine oocytes undergoing ICSI and activated by treatment with 7% ethanol for 7 min develop to the blastocyst stage (8).

Little has been known regarding the use of electric stimulation to activate bovine oocytes. Behalova *et al.* (20) noted that when bovine oocytes were stimulated by a single pulse of direct current, a nuclear membrane began to form immediately after the electric shock. In the human, electric stimulation was helpful to a woman whose husband's spermatozoa failed to fertilize after conventional ICSI (21). The current study was performed to determine whether bovine oocyte injected with single spermatozoon could be activated and sustain cleavage and further development after electric stimulation. We noted that 2PN formation of B&A ICSI treatment group were significantly higher than other groups. Cleavage rate was not statistically different among treated groups; however, morulae and blastocyst formation of the B&A injection group was significantly higher and one became pregnant. Although the exact mechanism is yet to be elucidated, electric stimulation before and after injection seems to enhance oocyte activation and embryo development in the bovine ICSI.

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